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Original Article

# Influence of insulin and testosterone on diabetic rat ventral prostate: Histological, morphometric and immunohistochemical study

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ABSTRACT

Many studies have reported high prevalence of hypogonadism amongst the diabetic patients suggesting the therapeutic benefits of testosterone hormone administration to these patients. The present research was carried out to study the possible role of insulin, testosterone and combined insulin and testosterone therapy on the diabetic rat ventral prostate. Fifty-eight adult male albino rats were used and divided into 2 main groups; the control and the experimentally induced diabetic group that given a single intra-peritoneal injection of STZ (60 mg/kg). The rats with confirmed DM were subdivided into: diabetic group that kept without treatment, diabetic–testosterone group (100 mg/kg/4 weeks, S.C.), diabetic–insulin group (5 IU/day/rat, S.C.) and diabetic–insulin–testosterone group. After 8 weeks, the ventral prostatic lobes were dissected and processed for paraffin blocks to study the epithelial height, mast cell number, expression of AR, PCNA and active caspase-3. DM induced a significant decrease in mast cells number, epithelial height and androgen receptors (AR) with a significant increase in the apoptotic index. On the other hand, the epithelial cells showed a non-significant change in the proliferation rate compared to the control group. Treatment with testosterone induced a significant increase in the epithelial height, number of mast cells & AR positive epithelial cells and a non-significant increase in PCNA with a significant decrease in the active caspase-3. Treatment with insulin showed abundant mast cells in the stroma with a significant increase in epithelial height & AR with decrease in active caspase-3 positive cells and insignificant changes in PCNA. Combined treatment with both hormones produced similar outcomes to the group treated with insulin alone. It was concluded that diabetes leads to alterations in rat ventral prostate gland. Early insulin treatment attenuates these effects with preserving most of the measured parameters within the normal values. Furthermore, addition of testosterone to insulin did not seem to produce a significant therapeutic add.



## 1. Introduction

Diabetes mellitus (DM) is a worldwide common disease. Its prevalence has been increased dramatically in recent years. Several investigators have conveyed the complications of DM on the structure and functions of the sexual and reproductive organs. Impotency, reduced libido, impairment of spermatogenesis, reduced sperm count and motility, decreased seminal fluid volume and decrease serum testosterone have been reported in diabetic men and in experimental animals [1]. The prostate gland depends on different hormones such as androgens, estrogens and prolactin. The testosterone is the main androgen that is

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very important for the development of prostate and maintenance of its structural and functional integrity. The subtle change in the testosterone level is usually accompanied by alteration in the growth and weight of the prostate [2]. The rat prostate contains a significant number of mast cells, with their density being higher during the pubertal period and declining significantly with age [3]. In the normal prostate, androgens act through a paracrine process with different effects on different types of cells. Binding of androgen receptor by its ligand results in dimerization and binding of the receptor to regulatory sequences of specific genes in the nuclei of epithelial and stromal cells [4]. This work was performed to study the effect of streptozotocin-induced diabetes on the prostate gland in adult rats and to study the role of insulin alone, testosterone alone and their combination in ameliorating the diabetic effect on the prostate.

## 2. Materials and methods

Fifty-eight adult male albino rats weighing 226–255 g each were used. They were kept in clean ventilated cages and fed on a commercial diet at the animal research house of Strathclyde University, UK under a controlled environment. They were divided into the following groups: A – *The control group (C)*: included 16 rats that were subdivided into 2 equal subgroups: the first was kept without treatment, the second received a single intraperitoneal (i.p.) injection of 0.2 ml of 0.1 M sodium citrate. B – *The streptozotocin induced diabetic group*: included 42 rats that were subjected to a single i.p. injection of 60 mg/kg streptozotocin (STZ) dissolved in sodium citrate just before injection [5]. Three days after STZ injection, the blood glucose levels were estimated using a blood glucose meter (Accu-Check Advantage, Roche, Germany). Only rats with blood glucose level of (≥250 mg/dl) were considered diabetic [6]. In this study, only 32 rats were proven to have DM, they were divided into 4 subgroups (8 rats each): 1 – *The diabetic group (D)*: that received no additional treatment. 2 – *The diabetic–testosterone group (D + T)*: that received testosterone undecanoate 100 mg/kg b.w./day, S.C. [7]. 3 – *The diabetic–insulin group (D + I):* that received human insulin (Humulin I), 5 IU/rat/day, S.C. [6]. 4 – *The diabetic–insulin–testosterone group (D + I + T):* that received insulin plus testosterone simultaneously as described above.

After 8 weeks, the animals were anaesthetized by intraperitoneal injection of sodium pentobarbital in a dose of 30 mg/kg b.w. [8]. The ventral lobes of the prostate gland of all animals were dissected and their middle areas were taken and processed for paraffin sections which were stained by toluidine blue for study of mast cell number and epithelial height and used also for immunohistochemical study of androgen receptor, caspase-3 and proliferative nuclear antigen (PCNA).

### 2.1. Immunohistochemistry

The sections were collected on APES coated slides, heat-mediated antigen retrieval was performed in pressure cooker with 0.1 M sodium citrate buffer (pH 6.0). The endogenous peroxidase was inactivated by 3% $H_2O_2$ in methanol for 30 min. The sections were treated with bovine serum albumin and normal goat serum in TBS as a blocking buffer for 1 h at RT then incubated overnight in a humidity chamber at 4 °C with primary antibodies (Polyclonal rabbit anti-Active caspase-3/ab1:300) and (Rabbit anti-Androgen receptor 1:100). On the next day, the sections were rinsed in 1% TBS–Tween-20 and TBS and incubated for 1 h at RT with the secondary antibodies (biotinylated goat anti-mouse and anti-rabbit/1:200). Avidin–biotin-complex reagent was added followed by incubating the sections with 3,3-diaminobenzidine (DAB) as a chromogen with Mayer's hematoxylin as a counter-stain then mounted with DPX. Negative control sections were obtained by removing the primary antibody and replaced by blocking solution [9].

### 2.2. Immunofluorescence study

Monoclonal mouse anti-PCNA (1:1000) as well as secondary antibody (Alexa Fluor 488 goat anti-mouse IgG) were used followed by vectashield mounting medium with DAPI (diamidino-2-phenylindole) [9] then examined and photographed by a fluorescent microscope.

### 2.3. Morphometric studies

The *epithelial height* was measured by a straight line extending from the basement membrane to the top of the tall epithelial lining of the middle area of the ventral gland described as a percentage of the control values and the *mast cell number* was quantified by counting the number of toluidine blue positive cells, both at ×400 field magnification using 15 random fields from 5 different rats in each group using image J software. AR, PCNA, and active caspase-3 were expressed as a percentage of total cell count (the number of positive epithelial cells × 100/total number of epithelial cells) by using 10 random fields from 5 different rats in each group using image J software at ×400 for AR, active caspase-3 and at ×200 for PCNA). All of these parameters were done at Histology Department, Faculty of Medicine, Tanta University.

### 2.4. Statistical analysis

All the values were expressed as the mean ± standard error of mean. Data was statistically analyzed using one-way ANOVA followed by Tukey's post-test. $p$-Value ≤0.05 was considered statistically significant. All statistical analyses were performed using Prism 5 software.

## 3. Results

Two rats were found dead after 6 weeks of treatment, one rat from the (D + I) group and the other from (D + I + T) group.

### 3.1. Light microscopic and morphometric results

Examination of paraffin sections stained with toluidine blue which is specific for mast cells revealed in the control group (C), many mast cells with metachromatic

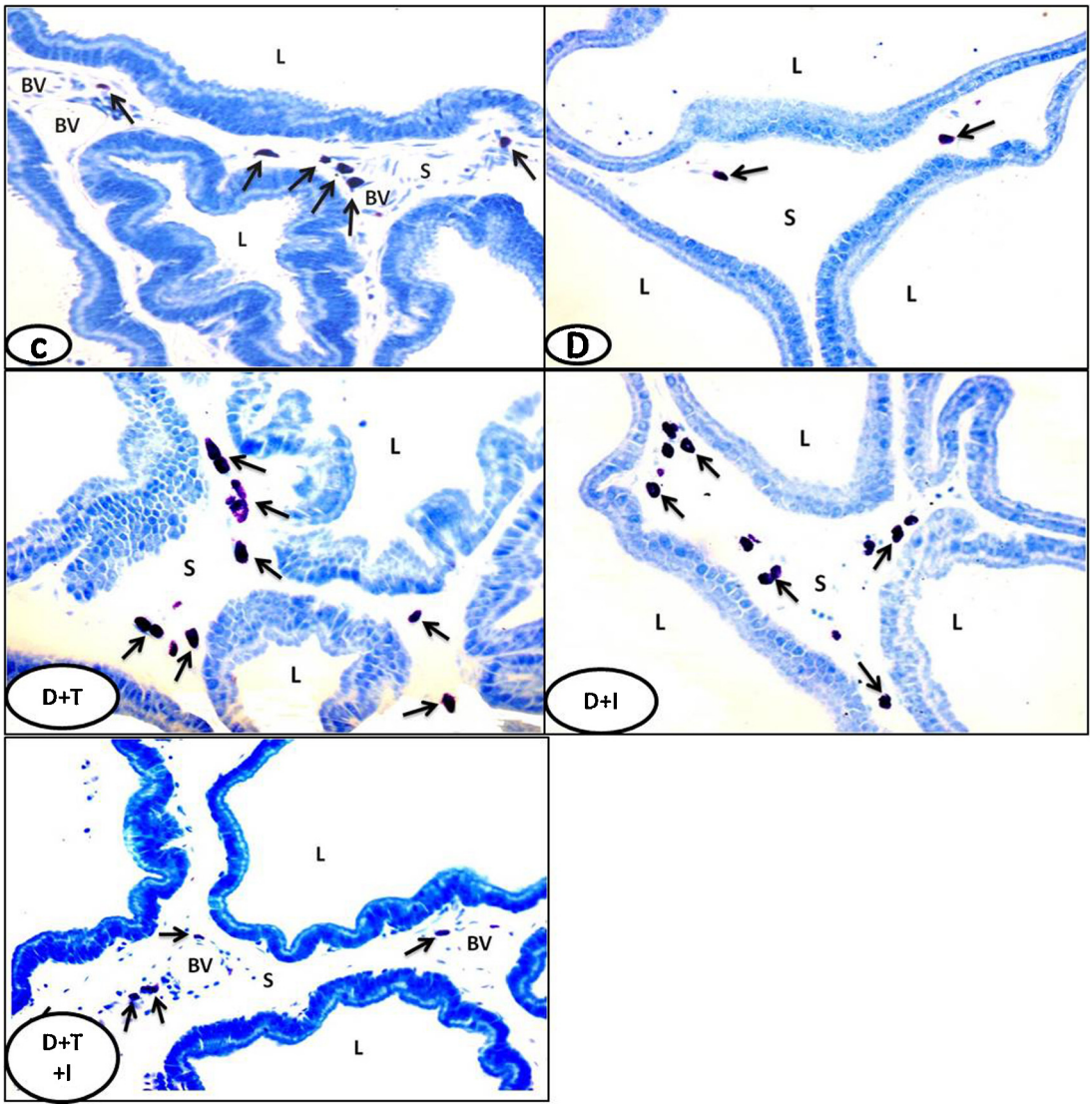


**Fig. 1.** Showing many mast cells (arrows) in the stroma of the control group (C) and few mast cells in the diabetic group (D). While, testosterone treatment (D + T) and combined hormonal treatment (D + T + I) showing many mast cells. Insulin (D + I) treatment showing abundant mast cells scattered singly or in groups. L: lumena; S: stroma; BV: blood vessels (toluidine blue ×400).

granules stained violet/red purple. These mast cells were localized in the stroma between the prostatic acini often close to the blood vessels. The diabetic group (D), showed apparent few mast cells in comparison to the control and the diabetic–testosterone group (D + T), showed apparent increase in the number of mast cells with apparent epithelial hyperplasia. Furthermore, there are abundant mast cells in the stroma of the sections obtained from the diabetic–insulin group (D + I), that appeared scattered or in groups. The diabetic rats received combined hormonal treatment (D + T + I), showed many mast cells of variable sizes in the stroma between the acini (Fig. 1).

*Morphometrically*: there was a significant decrease in the epithelial height lining the prostatic acini of the untreated diabetic group compared to that of the control group. While the epithelial height of the diabetic groups treated with testosterone, insulin and testosterone plus insulin displayed significant higher values compared to that of the untreated diabetic group (Table 1 and Fig. 2, Histogram A). The mast cell number in the ventral prostate confirmed the microscopic results and revealed a significant decrease in the number of mast cells in diabetic rats in comparison with that of the control rats. On the other hand, there was a significant increase in the number of mast cells with testosterone treatment compared to that of untreated diabetic group. While diabetic–insulin group showed a significant increase in the mast cells compared to all other groups (the diabetic, the diabetic–testosterone and the control groups). There was a significant increase in mast cell's number in diabetic rats treated with insulin plus testosterone in comparison with that of the diabetic group (Table 1 and Fig. 2, Histogram B).

**Table 1**
The average values of the epithelial height, mast cell number/field, AR, PCNA and active caspase-3 of the experimental groups.

| Groups | Epithelial height | Mast cell | AR | Active-caspase-3 | PCNA |
|---|---|---|---|---|---|
| C | 100 ± 3.3 | 4.4 ± 0.52 | 78.09 ± 1.8 | 0.4785 ± 0.1112 | 8.391 ± 0.8 |
| D | 63.88 ± 2.6*** | 2.04 ± 0.25*** | 50.81 ± 2.5*** | 2.407 ± 0.5796*** | 6.871 ± 0.8 |
| D+T | 99.13 ± 3.9*** | 6.0 ± 0.44*** | 96.29 ± 0.9*** | 1.400 ± 0.2142*** | 9.830 ± 1.8 |
| D+I | 95.97 ± 4.7*** | 8.13 ± 1*** | 76.43 ± 4.4*** | 0.5374 ± 0.1293*** | 7.365 ± 1.0 |
| D+I+T | 85.46 ± 2.4*** | 6.6 ± 0.84*** | 86.09 ± 3.5*** | 0.6859 ± 0.1162*** | 8.1 ± 1.034 |

*** $p < 0.0001$.

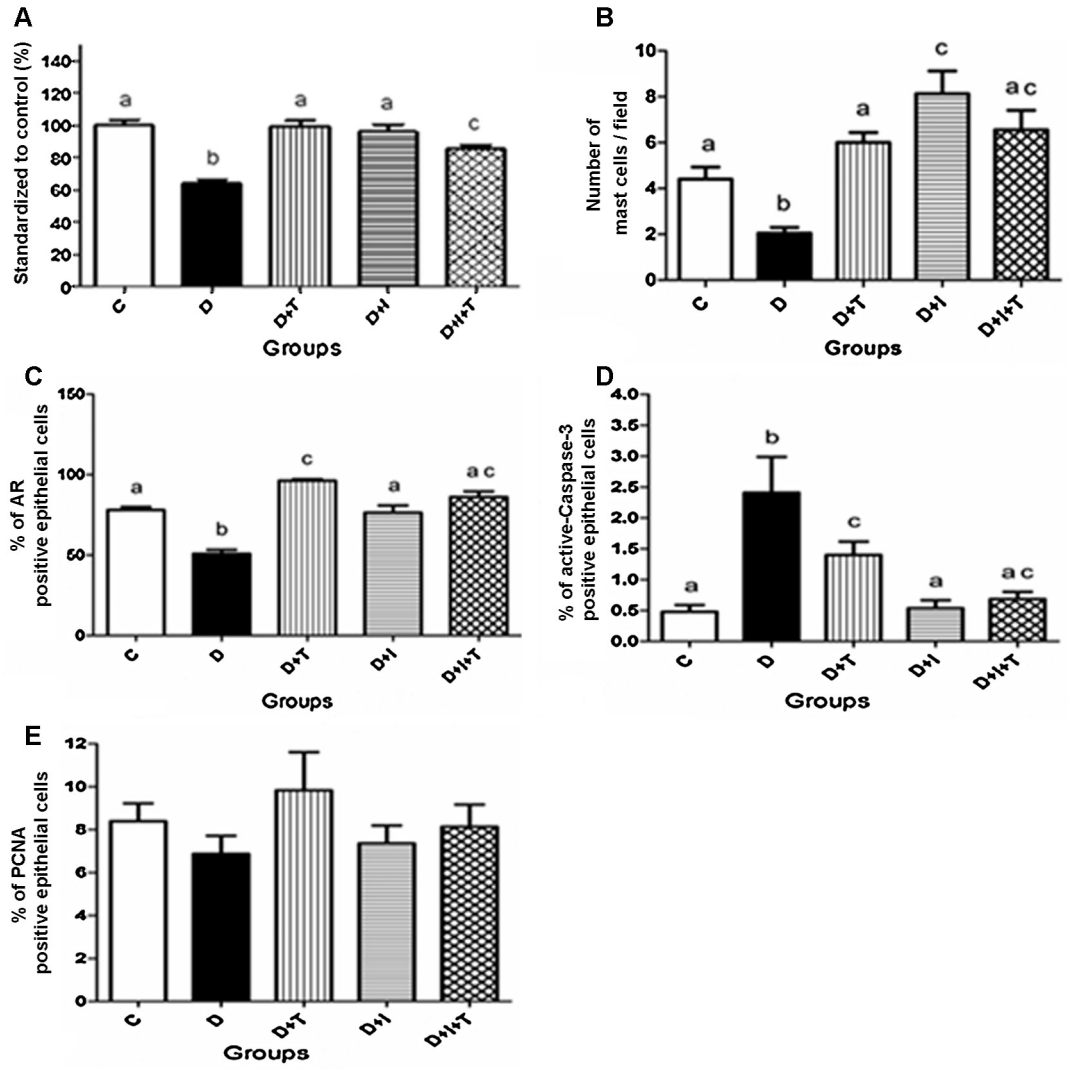


**Fig. 2.** Histogram A showing measurement of epithelial heights of rat ventral prostate of the experimental groups: (C) control; (D) untreated diabetic; (D + T) diabetic treated with testosterone; (D + I) diabetic treated with insulin; (D + I + T) diabetic treated with insulin plus testosterone. Histogram B shows quantification of mast cells. Histograms C, D and E show quantification of the androgen receptor positive epithelial cells, quantification of active caspase-3 positive epithelial cells (apoptotic index) and quantification of proliferative cell nuclear antigen positive epithelial cells (proliferative index) respectively in all the experimental groups. Different letters indicate significant differences.

### 3.2. Immunohistochemical results

*Androgen receptor (AR):* specificity of antibody reaction was confirmed by the absence of staining either in the nuclei or the cytoplasm of the negative control. The control group showed a positive immunoreactivity which was labeled as a brown color in the nuclei of the epithelial and stromal cells of the prostatic acini. However, the immunostaining of AR was apparently more intense in the epithelial cells than stromal cells. The untreated diabetic group showed apparent decrease in the number of the positive epithelial cell nuclei compared to control group.

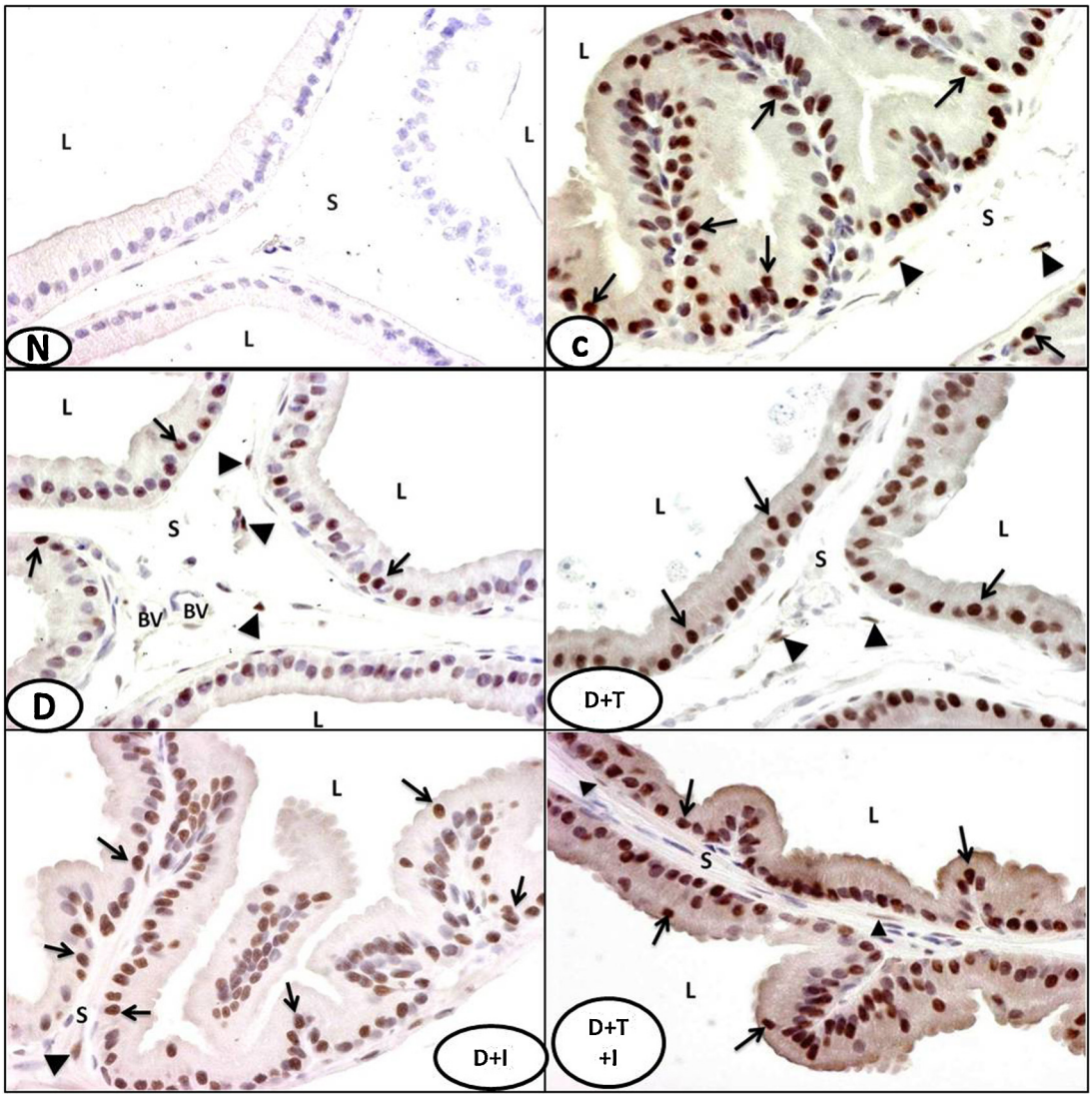


**Fig. 3.** Negative control (N) for AR immunostaining showing no immunoreaction in the cytoplasm or nucleus either in the glandular epithelial cells or stromal cells. Control group (C) shows many positive nuclear immunoreaction (arrows) for AR in the glandular epithelial cells and few immunoreactions in the stromal cells (arrow heads). Diabetic group (D) shows apparent decrease in number of positive immunoreaction (arrows) in the epithelial cells with some positive stromal cells (arrow heads). Testosterone treated group (D + T) shows positive nuclear immunoreaction (arrows) nearly in all epithelial cells with some positive stromal cells (arrow heads). Insulin treated group (D + I) shows many positive immunoreaction (arrows) in epithelial cells with some positive immunoreaction (arrow heads) in the stromal cells. Combined hormonal treatment (D + T + I) shows positive nuclear immunoreaction (arrows) in most of the epithelial cells. L: lumena; S: stroma; BV: blood vessels (AR immunostaining ×400).

The diabetic rats treated with testosterone showed apparent increase in the number of AR positive epithelial cells, almost all nuclei of the epithelial cells showed a positive immunoreaction of the AR. The treatment with insulin also showed apparent increase in the number of positive nuclei than that of diabetic group but less than those treated with testosterone. Furthermore, the combined hormonal therapy with insulin plus testosterone showed apparent increase in the AR immunopositive nuclei in the epithelial cells compared to the untreated diabetic rats (Fig. 3).

The statistical results showed a significant decrease in the percentage of AR positive epithelial cells in the diabetic group compared to the control group. There was a significant increase in the percentage of AR positive cells in the diabetic animals treated with testosterone, insulin and combined insulin plus testosterone in comparison to that of the untreated diabetic rats. In addition, diabetic–insulin group showed a significant decrease in the percentage of AR positive epithelial cells compared to the diabetic–testosterone group but no significant differences were reported in the percentage of the AR immunoreaction in combined hormonal-treatment group compared to the insulin treated diabetic group (Table 1 and Fig. 2, Histogram C).

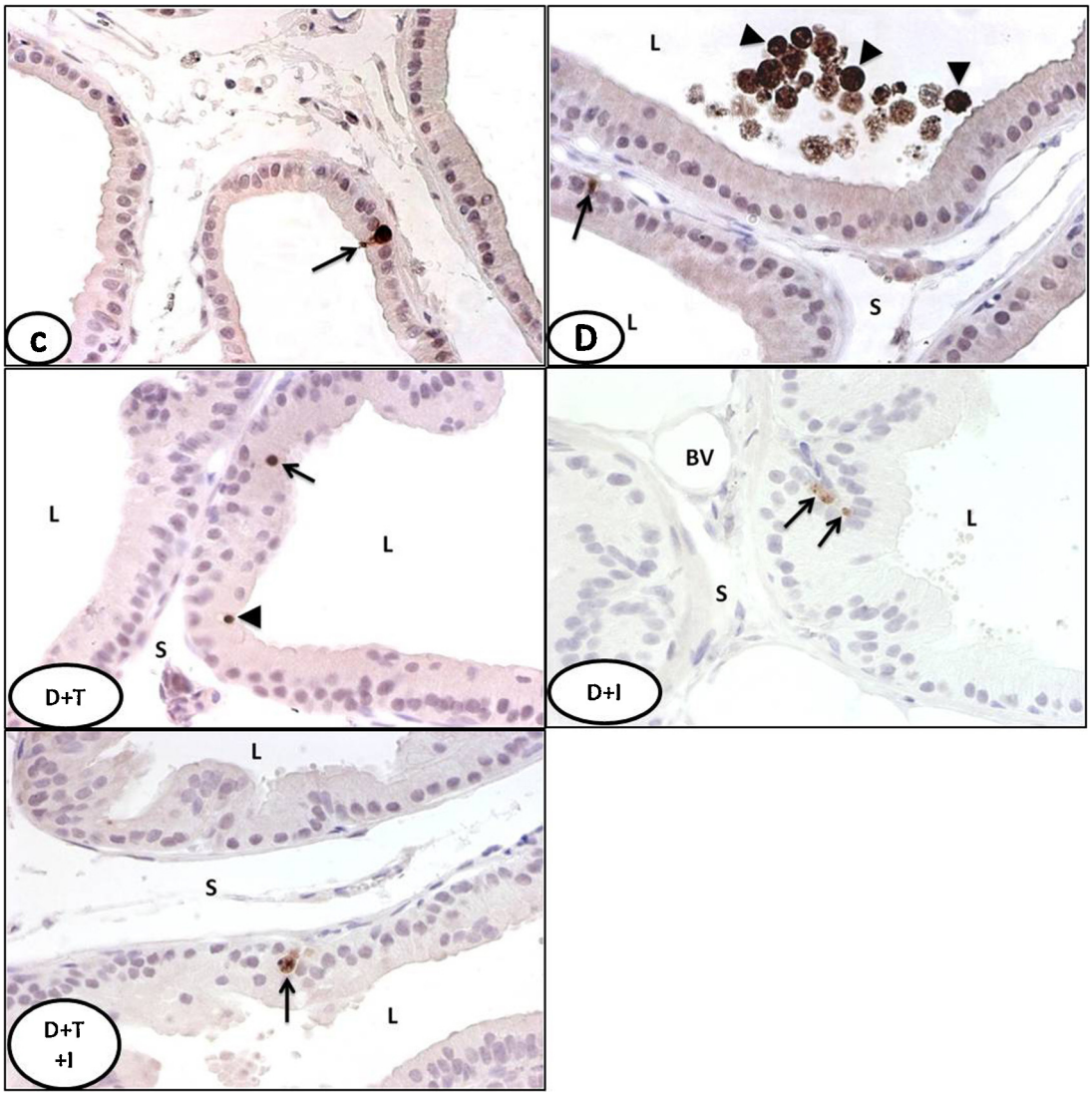


**Fig. 4.** Showing localized positive cytoplasmic immunoreactions (arrows) in the glandular epithelial cells in control group (C), accumulation of many positive cells (arrow head) in the lumen of the acini in diabetic group (D), localized positive nuclear immunoreaction (arrows) in some nuclei surrounded by vacuolated cytoplasm (arrow heads) in testosterone treated diabetic rats (D + T), localized positive cytoplasmic immunoreaction (arrows) in the glandular epithelium in insulin treated (D + I) and combined hormonal treated (D + T + I) diabetic rats. L: lumena; S: stroma; BV: blood vessels (active caspase-3 immunostaining ×400).

### 3.3. *Active caspase-3 (an apoptotic marker)*

Immunohistochemical staining of the sections obtained from the control group showed a little active caspase-3 immunoreactivity in the cytoplasm of glandular epithelial cells. The diabetic group showed increased number of positive epithelial cells compared to control group. The anti-active caspase-3 was localized in the cytoplasm as well as in the nuclei of cells. The majority of the reactions were noted in the epithelial cells during process of separation from the acini and in the already separated cells in the lumen. There was apparent decrease in the expression of the active caspase-3 in the diabetic–testosterone group. Furthermore, the diabetic–insulin group and that with combined hormonal treatment showed a few positive cytoplasmic immunostaining of active caspase-3 in the epithelial cells (Fig. 4).

Expression of the active caspase-3 in the epithelial cells of the ventral prostate was quantified as an apoptotic index. It was calculated by dividing the number of epithelial cells with positive reaction of active caspase-3 by the total number of the epithelial cells. Our results showed a highly significant increase in the apoptotic index in the diabetic group compared to the control group. On the other hand, the diabetic–testosterone group showed a significantly lower average value of apoptotic index than those found in the diabetic group. However, it was significantly higher than that of the control group. The diabetic–insulin group showed a significant low average value of the index compared to the diabetic group and it

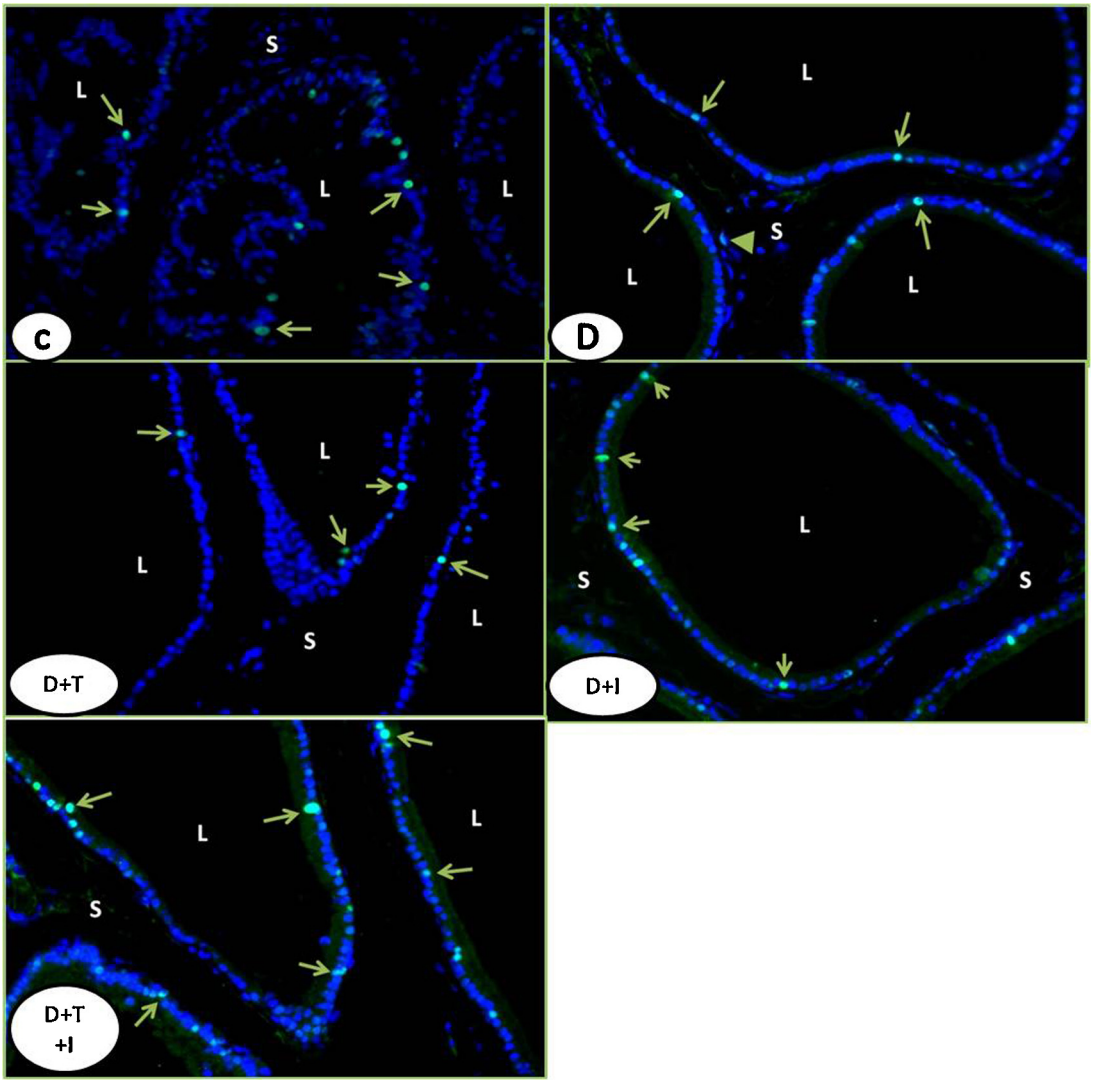


**Fig. 5.** Showing nuclear localization of PCNA immunostaining in the nuclei of some glandular epithelial cells (green arrow) and positive nuclear immunostaining (blue color) for DAPI in the glandular epithelial cells in control (C), diabetic (D), testosterone treated (D + T), insulin treated (D + I) and combined hormonal treatment (D + T + I). L: lumena; S: stroma (immunofluorescence ×200).

was insignificant compared to the control group. Whereas the diabetic–insulin–testosterone group showed a highly significant decrease in the index compared to the diabetic group, with no significant difference compared to other experimental groups (Table 1 and Fig. 2, Histogram D).

### *3.4. Immunofluorescent study of proliferating cell nuclear antigen (PCNA)*

The merge images of both DAPI and PCNA immunoreaction of the same section confirmed the co-localization of PCNA positive immunoreaction and the DAPI staining denoting nuclear localization of the PCNA in the glandular epithelium of all experimental groups. The immunostaining for PCNA in the control group showed positive nuclear reaction in some acinar epithelial cells. This reaction appeared as a green coloration of the nuclei of the positive cells. The diabetic group showed apparently few positive nuclear immunoreaction in glandular epithelial cells (secretory tall columnar cells) with positive immunoreaction in the stromal cells. The diabetic group treated with testosterone, displayed the same pattern of nuclear immunostaining for PCNA in the glandular epithelium with apparent increase in the positive nuclei compared to the control and the diabetic groups. However, the diabetic rats that were treated with insulin alone or combined hormones showed few positive nuclei for PCNA in the glandular epithelium (secretory tall columnar cells) (Fig. 5). The quantitative analysis of PCNA-labeling index in the glandular epithelium showed no significant variation in the proliferative activity between the different experimental groups (Table 1 and Fig. 2, Histogram E).

## 4. Discussion

Many studies have shown that between 20% and 64% of diabetic men are suffering from hypogonadism, with high prevalence in the elderly that can be related to decreased sex hormone binding globulin, decreased gonadotrophin release or testosterone production. On the other hand, hypogonadism could be a contributing factor to diabetes due to the changes in the body composition, androgen receptor polymorphisms, glucose transport and reduced level of the antioxidants [10].

In this study, there was marked epithelial atrophy in the glandular epithelium of diabetic group that was in agreement with a previous work [11] which showed a reduction in the glandular epithelial height and the volume of the epithelium in experimental diabetes. The prostatic atrophy in diabetes is similar to the prostatic involution following castration, which is due to the selective loss of the androgen-dependent secretory epithelial cells due to apoptosis and is accompanied by a marked reduction of both the cytoplasmic and nuclear androgen receptor content. These changes can be prevented by the administration of androgens [12] that reflect the anabolic effect of testosterone and its role in cell proliferation and stromal growth [13]. The growth-promoting effects of insulin may be due to its role in stimulating the production of testosterone by affecting the hypothalamic–hypophyseal–testes axis as well as its local effects through insulin receptors [14]. In addition, some stated that blocking insulin production during sexual maturation compromises prostate growth [15].

Some workers [16] had reported that prostatic mast cell degranulation and histamine release may induce smooth muscle contraction in the prostate. This finding may clarify the accumulation of the mast cells in the stoma near the smooth muscle and the glandular epithelium. Mast cells are often observed in close relation to the blood vessels that justifies the involvement of mast cells in the angiogenesis and the vascular control of the prostate [17]. The present work showed a significant decrease in the number of mast cells in diabetic group, this was observed previously by reporting a severe reduction in number of the mast cell in different body sites such as mesentery and intestine under the diabetic condition [18]. Others [19] reported also a decrease in the number of pleural mast cells in alloxan-induced diabetes. The diabetes is associated with an increase in the serum glucocorticoid which plays a role in the depletion of mast cells from different body sites as skin, lung and intestine [20].

In this study, treatment with testosterone, insulin or combined hormonal therapy was associated with an increase in the number of the mast cells. Some authors [21] observed that surgical adrenalectomy and treatment with steroid receptor antagonist completely reversed the mast cell depletion observed under the diabetic conditions. Others reported accumulation of the mast cells in the rat prostate during the first day of testosterone treatment [22]. Some investigators reported an increase in the pleural mast cells of diabetic rats after insulin treatment [23]. Similar findings showed that insulin might modulate mast cell degranulation at the early-phase response to antigen provocation decreasing the risk of asthma among type-1 diabetic patients. This also could be attributed to the modification in the level of corticosteroids [24].

The distribution of AR varied in the different cells of rat ventral prostate, the nuclear staining for AR in the epithelial cells was stronger than that of the stromal cells and these verities in the expression of AR in different types of cells of rat ventral prostate suggest that these cells have different AR contents and different responses to androgen [25]. In this study, there was a significant decrease in the androgen receptor expression in the diabetic rats. This finding was in line with other studies that stated that the intensity of AR reaction was lower one week after diabetes when compared to respective controls and they related that to the lower testosterone level in diabetic state [6]. In the same time, they also stated that three months after alloxan-induced diabetes, there was no changes in the frequency of AR-positive cells in the prostatic acinar epithelium compared to their age matched control group and related that to the already low level of testosterone in older rats. It was stated that the expression of AR mRNA decreases in testis, epididymis and prostate of diabetic rats, that weakening the biological effects of AR and it might be one of the causes responsible for the sexual and reproductive dysfunction in the diabetic rats [26]. In this study, the diabetic rats received treatment with insulin and testosterone showed significant increase in the AR expression, these observations were in line with the ability of combined insulin and testosterone to restore the weight of the accessory sex gland with partial structural recovery [27].

However, in this study, there was a positive immunostaining for active caspase-3 in the control rats that was supported by some authors who revealed the positive active caspase-3 immunoreaction in the epithelial cells of prostatic acini from the control rats [28]. The quantitative analysis of apoptotic index showed that the apoptotic cells were more abundant in diabetic rats especially in detached epithelial cells in the acinar lumen. Some researchers confirmed that diabetes stimulates apoptosis in different organs including the prostate [29]. Additionally, it has been reported that the apoptotic rate in the ventral prostate of the diabetic group increased with the disease progression which suggests that these alterations might be due to low testosterone level that reported in diabetes as a potential cause for the increased apoptosis in the diabetic tissues [30]. As well as the increase in the oxidative stress condition with the reduction in the level of the antioxidant enzymes in diabetes directly induce apoptosis by damaging the DNA [31]. The anti-apoptotic role of testosterone may contribute to the ability of testosterone to up regulate the expression of anti-apoptotic genes such as bcl-2 [32]. It was reported that insulin administration reduced the overall rate of apoptosis in the presence and absence of androgen stimulation [11].

Proliferating cell nuclear antigen (PCNA) expression usually used to discriminate cycling from non-cycling cells and is localized to the nuclei of the proliferating cells [33]. In this study, the results revealed no significant variations

in cell proliferation rates amongst the experimental groups after 8 weeks in the diabetic state. These findings were also observed after one week in the diabetic state as there was a notably low intensity of AR that resulted in the decrease in the growth factors (released from the stromal cells) with consequent impairment of the cellular proliferations [6]. While, others observed a marked reduction in the cell proliferation levels in the acinar epithelium of the ventral prostate after one month of diabetes [28].

Furthermore, previous reports about testosterone supplementation in diabetic animals revealed that the androgen does not completely establish changes in the reproductive system, while insulin replacement can reverse the prostatic involution and serum level of testosterone [34]. In another recent study, administration of insulin, testosterone and estrogen alone did not restore the balance between the proliferation and apoptosis in non-obese diabetic mice. While, the combined administration of the three hormones produced morphological recovery of the prostate [35].

## 5. Conclusion

Early diagnosis of type I diabetic patients and treatment with the optimal type and dose of insulin are recommended. Great precautions should be taken with long acting testosterone with restriction of its application only to the cases suffering from clear hypogonadism associated with diabetes mellitus. Further studies are required to better understand the role of insulin and testosterone in maintaining the morphology and the function of the prostate gland and other reproductive organs.

## Conflict of interest

None declared.

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